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The overall goal of this research project was to study the mechanisms by which non-genotoxic chemicals induced multiple disease end points such as birth defects, tumor promotion, reproductive- and neuro-toxicities. The working hypothesis was that these non-genotoxic chemicals disrupted homeostatic control of cell proliferation, differentiation and adaptive responses of differentiated cells. Specifically, to test this hypothesis, gap junctional intercellular communication (GJIC) was studied. During this grant period, several techniques were developed and applied to study how various model non-genotoxic chemicals, as well as various oncogenes, interfered with GJIC to cause abnormal cell growth and differentiation. The molecular biology, biochemistry and cell biology of non-genotoxic chemical and oncogene interference with GJIC was studied using *in vitro* techniques, primarily with fluorescent detection of ions and molecules via laser-assisted image analyses. The major findings to date show (a) that inhibition of GJIC by non-genotoxic chemicals and a number of oncogenes correlates with their toxicities; (b) new techniques to measure GJIC have been developed and validated; (c) molecular/biochemical mechanisms underlying the regulation of GJIC are now being unraveled; and (d) new cell mutants/transfектants have been developed to study the mechanisms by which non-genotoxic chemicals might induce diseases.

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FINAL TECHNICAL REPORT
"THE ROLE OF GAP JUNCTIONAL COMMUNICATION
IN CHEMICAL TOXICOLOGY"

(F49620-92-J-0293)

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CONTENT

I. COMPREHENSIVE TECHNICAL SUMMARY OF SIGNIFICANT WORK ACCOMPLISHED

A. OVERALL GOAL OF RESEARCH

The members of the U.S. military are exposed to potentially toxic chemicals during both routine performance of their duties, as well as during specialized roles in training and action. Acute and chronic exposure to some of these chemicals could lead to serious health effects. The goal of this research was to study the mechanisms by which chemicals, known or suspected to be non-genotoxic, caused birth defects, cancer, or reproductive and neurological disorders. The hypothesis driving this research was that inference or inhibition of the process by which a body maintains homeostatic control of cell proliferation, cell differentiation or adaptive responses of differentiated cells, namely GAP JUNCTIONAL INTERCELLULAR COMMUNICATION, was the common cellular mechanism for non-genotoxic chemicals. In other words, to interfere with cell communication during early embryonic development could lead to birth defects; inhibition of cell communication in tissues which harbored "initiated" or premalignant cells, could lead to tumor promotion; inhibition of cell communication in the ovaries or testes would interfere with the maturation of eggs and sperm, leading to reproductive dysfunction; and finally, inhibition of cell communication in the brain and nervous system could lead to various neuro-toxic and behavioral effects.

B. SPECIFIC TECHNICAL APPROACH OR AIMS

The approach used was that of in vitro use of various rodent or human cells. The approach was to determine if various model, known epigenetic toxicants would block gap junctional intercellular communication (GJIC), using several methods to measure the functional status of gap junctions which were developed under the sponsorship of this grant. In addition, we also used current molecular and biochemical techniques to measure the gene and protein products related to GJIC under those conditions where the toxic chemicals inhibited GJIC. Moreover, molecular genetics techniques were used to study how various oncogenes, which code for various growth factors, growth factor receptors, signal transducing elements and transcription factors, also interfere with GJIC. The approach has set the stage for unifying two different fields of research, namely toxicology and cancer research. All of the techniques, equipment and

results have been published or are being prepared for publication in peer-reviewed scientific journals (see section 9).

C. OVERALL SUMMARY OF RESULTS UNDER THIS GRANT

1. Overall Summary of Results Under This Grant

Progress made in my laboratory on the role of gap junctional intercellular communication in tumor promotion, which started with our demonstration that the phorbol esters, a powerful skin tumor promoters, inhibited GJIC [Science 232:425-428, 1986] has evolved to include (a) our development of 4 different ways to measure the function of GJIC [metabolic cooperation assay; scrape-loading/dye transfer assay; fluorescent recovery after photobleaching ("FRAP"); and microinjection/FRAP], (b) identification of scores of chemicals which can inhibit GJIC and are known to be teratogens, tumor promoters, reproductive toxicants, and neurotoxicants []; (c) identification of a number of oncogenes (ras, arc, raf, neu) which also contribute to the down regulation of GJIC; (d) recent identification of several tumor suppression genes which up regulate GJIC; and (e) finally, the detailed biochemical mechanism by which two different classes of tumor promoters (e.g., phorbol esters, pesticides) block GJIC.

This last advance can best be summarized by stating that both the phorbol ester, TPA, and pesticide, DDT, quickly block GJIC at the posttranslational level by modifying the phosphorylation status of the connexin 43 protein. However, TPA, by activating protein kinase C, induces a hyperphosphorylated connexin 43. On the other hand, DDT causes a hypophosphorylated connexin 43 protein by an unknown mechanism. In both cases, whether hyper- or hypophosphorylated, the gap junction does not function. This demonstrates that not all tumor promoters work by the same biochemical mechanism, therefore chemopreventive measures must be designed to the particular underlying mechanism.

Along these lines, we were the first to demonstrate that by knowing the specific mechanism by which a specific oncogene blocks GJIC, specific drugs could be used to ameliorate the effect of the oncogene (32).

2. Development of Confocal Imaging to Study Effect of Oncogenes and Chemicals on the Trafficking of Gap Junction

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We had noticed that since various types of chemicals and oncogenes which inhibit GJIC do so by different mechanism, there might be clues as to how they down-regulated gap junctions (trafficking to membrane, assembly in the membrane, assembly in plaque in membrane; gating of the gap junctions etc.) by confocal imaging. Without the new Meridian Ultima, we have preliminary data showing a major difference in the cellular localization of the gap junction protein.

3. Regulation of Various Gap Junction Genes

While certain inhibitors of GJIC occur at the posttranslational level (e.g., TPA phosphorylations of connexin 43 protein), other modulators (oncogenes, growth factors, anti-tumor promoters such as retinoids and tumor suppressor genes) may be acting at the transcriptional or gene expression level. It is imperative, with molecular techniques available to identify the regulatory sites in the gene sequence of the major gap junction genes for transcription factors (DNA binding proteins) and methylation sites which could influence the expression of the gene.

We have started to characterize one connexin gene (human and rat connexin 43) for methylation sites to see if it correlates with GJIC in normal, premalignant or malignant human breast and rat liver cells.

We will need to clone the genomic human/rat for CX 26, CX 32, CX 43. [This is being done on my NCI grant]. The use of up-stream regulatory regions DNA sequences fused with reporter constructs such as CAT or Luciferase reporter genes will aid to identify DNA binding regulatory sites and restriction enzymes recognizing or resistant to DNA methylation will be used. For this project, only the DNA methylation site studies will be performed.

4. Restoration of GJIC in GJIC-deficient Tumorigenic Rat Liver Epithelial Cells

To determine the potential role of gap junctions in normal growth regulation, we transfected the rat connexin 43 gene into our GJIC-deficient mutant rat liver epithelial cell (isolated and characterized earlier in this grant project). We were able to restore GJIC in these cells, as well as to demonstrate that they had normal growth control in vitro. We will now need to determine if the tumorigenicity of this CX 43 restored cell line does or does not form tumors in the animal. This work was recently published (Molecular Carcinog. 8:234-244, 1993).

5. Role of GJIC in Grow-control of Rat Liver Epithelial Cells

One of the most important questions to be answered concerning the biological role of gap junctions is whether it is, indeed, necessary for normal cell growth control. Several published studies have provided circumstantial evidence in support of this idea. On the other hand, a few reports on different cells and under different conditions have suggested that only physical contact of cell membranes is needed or that exogenous negative growth regulators, but not physical contact or gap junctions are needed. In a recent case (64), both GJIC and exogenous factors were involved in a co-culture of normal GJIC+ and GJIC- tumorigenic cells.

Our recent studies with our normal rat liver epithelial cells (GJIC-) is cultured with a number of oncogene or mutated GJIC-deficient cells have clearly shown in our system that gap junctions are a necessary component in the regulation of cell growth control. A paper has been submitted.

6. The Role of Oncogenes in the Down Regulation of Gap Junction Function

To further test our hypothesis that both genes (oncogenes) and exogenous chemicals which influence carcinogenesis do so by inhibiting GJIC, we have continued to study the mechanism by which the neu-oncogene (c-erbB-2) interferes with GJIC. We have almost completed the study and have shown (1) that expression of the neu oncogene does reduce, significantly, GJIC when transfected into normal, communicating rat liver cells; (2) that these cells are tumorigenic when put back into the rat liver; (3) the gap junction protein is abnormally phosphorylated, indicating this oncogene affects GJIC at the posttranslational level, and (4) this abnormally-phosphorylated CX43 protein affects its trafficking such that the CX43 protein appears to be in the nucleus instead of the membrane. A manuscript is being prepared and further studies with confocal imaging will be done to answer the question if a trafficking problem. This observation could be a major clue as to how proteins, in general, are modified to be signals for directional trafficking in the cell.

7. Apoptosis and Gap Junctional Intercellular Communication

During this past grant period, we noticed in the literature that many tumor promoting chemicals, such as TPA, phenobarbital and DDT, could block apoptosis. These same chemicals could block gap junction

function. Further, oncogenes which blocked apoptosis also blocked GJIC, while several anti-tumor promoters (retinoids, dexamethasone) increased GJIC and increased apoptosis.

We have therefore formulated a working hypothesis (submitted to Molecular Carcinogenesis, Appendix I) stating that, in solid tissues, GJIC is necessary for apoptosis and by blocking apoptosis with chemicals, one could promote initiated premalignant cells or by increasing apoptosis by increasing GJIC, one could prevent tumorigenesis.

8. Summary of Overall Progress

To date, we now have a unique set of cell lines derived from a normal rat liver epithelial cell line, given to us by Dr. Joe Grisham (University of North Carolina). This line has been thoroughly characterized for normal growth, biochemistry, transformability, tumorigenicity. We now have isolated and characterized gap-junction-deficient clones; clones of ras; raf; src; mye; ras/myc; neutransfected cells, as well as obtained a lac-z marker WB clone. This latter cell will enable us to identify a single marker cell when either co-cultured. We now have a series of human keratinocyte, human kidney, human breast and rat and human brain-derived cells which all have functional GJIC and therefore can be used for in vitro comparative, species and organ differences. We also have clones for the three major rat and human connexin cDNA's, as well as antibodies to CX43, with the potential of antibodies to CX 26 and CX 32. We also have several tumor line transfected with CX's and the p53 gene, as well as normal cells transfected with the MDM2 gene (produces the MDM2 protein which binds to the p53 protein). Lastly, we now know there are different mechanisms by which various epigenetic toxic chemicals block GJIC. This knowledge should aid in potential chemoprevention strategies to ameliorate the toxic effects.

9. Published Papers During Past Grant Period

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ras T24 protein with extent of loss of gap junction function in rat liver epithelial cells. *Molec. Carcinogenesis* 5:205-212.

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10. Manuscripts Submitted or in Preparation

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2. Hshu, H., Trosko, J.E. and Madhukar, B.V. (1992) Xenobiotic inhibition of intercellular communication in rat leydig cells in vitro (in preparation for *In Vitro Toxicol.*).
3. Paradee, W.J., Madhukar, B.V. and Trosko, J.E. (1992) Mezerein inhibition of intercellular communication in human kidney epithelial cells: Correlation with sustained membrane association of protein kinase C (in preparation for *Biochem. Biophys. Res. Commun.*).
4. Madhukar, B.V., Oh, S.Y., Quensen, J., Wade, M., Jiwa, A.H. and Trosko, J.E. (1992) Inhibition of intercellular communication and regulation of intracellular calcium by dieldrin, heptachlor and heptachlorepoxyde in a rat liver epithelial cell line in vitro (in preparation for submission to *J. Cell. Physiol.*).
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mezerein in rat liver epithelial cells (in preparation for submission to *Carcinogenesis*).

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11. List of Abstracts of the Work Presented at Meetings

1. Trosko, J.E., C.C. Chang and B.V. Madhukar. 1989. In vitro analysis of modulators of intercellular communication: Implications to mechanisms of tumor promotion and to predictions of potential tumor promoters. 2nd Intern. Conf. on Practical In Vitro Toxicology, Nottingham, U.K., July 23-27, 1989.

2. Trosko, J.E., J.E. Klaunig, B.V. Madhukar, C.C. Chang, E. de Feijter and G. Kalimi. 1989. Chemical and oncogene modulation of intercellular communication during carcinogenesis. Symposium on Molecular Cell Biology of Liver Growth and Function, Lake Placid, NY, Aug. 13-16, 1989.
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II. TRANSITION REPORT

- A. These studies contributed to the validation of the concept that inhibition of gap junctional intercellular communication by chemicals can be used as an indication of potential toxicity of the chemical.
- B. We have developed several *in vitro* techniques to measure gap junctional intercellular communication in order to minimize the use of animals for toxicity studies and to use both animal and human cells for comparative toxicological purposes.
- C. We showed that these classes of chemical toxicants could block GJIC at the gene level, as well as the level of protein synthesis, assembly of the gap junction channel and of the gap junction function.

This was done because, in order to predict the potential of a given chemical to be a toxicant (for risk assessment purposes), basic molecular and biochemical mechanisms by which toxic chemicals could block GJIC must be understood.

- D. The use of oncogenes (gene which influence the cancer process) and known chemical toxicants, which have been shown to cause birth defects, growth of pre-malignant lesion (tumor promoters),

reproductive dysfunction and neurological disorders, showed that gap junction function was altered.

- E. Toxic chemicals had differential effects on GJIC depending on the (a) specific gap junction gene expressed in a cell; (b) physiological or differentiated state of the cell; (c) concentration of the chemical to which the cell was exposed (IMPORTANT BECAUSE IT IMPLIES THAT THIS CLASS OF TOXIC CHEMICALS HAVE THRESHOLD LEVELS IN ORDER TO BE TOXIC); and (d) mixtures of chemicals could be additive, antagonistic or synergistic in their effects on GJIC.
- F. Our studies contribute to the understand that those toxic chemicals which act by inhibiting intercellular communication are, indeed, non-genotoxic (non-mutagenic or non-DNA-damaging). These chemicals are, indeed, acting by epigenetic mechanisms (that is, they altered the expression of genes (not the information of genes) at the transcription level (turning genes on or off), translational level (stabilize or de-stabilize gene's messages), or posttranslational levels (modifying the proteins coded by genes).

This implies that a one-time exposure, or even long-term exposure to sub-threshold levels of this class of chemicals will either be reversible or non-health threatening.

- G. Clearly, our studies to date suggests that toxic chemicals of the class which blocks GJIC acts as natural endogenous chemicals (hormones, growth factors, neurotransmitters) via stimulating or altering signal transduction in cells.
- H. The new proposal for the next three years is designed to further delineate, on the molecular and cellular levels, how these class of chemical toxicants work and to apply the concepts and techniques to characterize a number of chemicals to which the Air Force personnel and their environs must be exposed.
- I. Lastly, to put this into perspective, gap junctional intercellular communication is a fundamental and absolutely vital biological process to maintain health in a human being (homeostasis). Intercellular communication of cells, within and between tissues, helps to control the growth of cells, their ability to differentiate and when differentiated, their ability to respond properly to changes in their environment. Chemicals which block gap junctional intercellular communication will disrupt homeostasis, thereby cells lose their ability to control cell growth, do not differentiate properly

and can not respond correctly, when they are differentiated, to changes in their environment.